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Isolation of a Soluble Cadmium-Binding Protein from Pulmonary Macrophages¹

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Isolation of a Soluble Cadmium-Binding Protein from Pulmonary Macrophages. Cox, C. C., AND WATERS, M. D. (1978). *Toxicol. Appl. Pharmacol.* 46, 385-394. A soluble cadmium-binding protein, with properties similar to metallothionein, has been isolated from rabbit alveolar macrophages. The macrophages were cultured in Medium 199 with Earle's salts for 24 hr in the presence of $10 \mu\text{M}$ CdCl_2 and carrier-free ^{109}Cd as a tracer. The isolation procedure began with application of a 100,000 g cell supernatant to a column of Sephadex G-75 Fine. The fraction containing the greatest amount of cadmium was eluted at a relative elution volume, V_e/V_o , of 1.87. A molecular weight determination performed following Sephadex chromatography indicated that the apparent molecular weight of the impure protein was approximately 11,000. The fractions containing cadmium were pooled and purification procedures were applied, including acetone fractionation, DEAE-cellulose chromatography, and polyacrylamide gel electrophoresis. DEAE-Cellulose chromatography following acetone fractionation indicated the presence of two forms of metalloprotein as has been demonstrated previously in the isolation of cadmium-thionein from liver and kidney. The two forms of metalloprotein were subjected to polyacrylamide gel electrophoresis and, although separation was incomplete, bands obtained corresponded to those typically observed in rat liver.

Metallothionein, a divalent metal-binding protein, was first isolated by Margoshes and Vallee (1957) and characterized by Kagi and Vallee (1960, 1961). Since that time both liver and kidney metallothioneins have been well characterized by many other investigators (Nordberg *et al.*, 1974; Piscator, 1964; Webb and Daniel, 1975; Webb, 1975; Wesser *et al.*, 1973; Winge *et al.*, 1975). In 1964, Piscator proposed that the cadmium metallothionein is involved with detoxification of cadmium, mercury, and other divalent metals. However, in 1975, Webb suggested that metallothionein is involved primarily in the regulation of zinc metabolism and that the detoxification property of the protein is secondary.

Recently, interest has developed in determining whether metal-binding proteins are present in organs other than liver and kidney, particularly in the lung. Cadmium has been mentioned as one of the metals which tend to be concentrated in the small

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respirable particle size fractions from ambient air (Natusch *et al.*, 1973). Furthermore, it has been shown that there is an increased cadmium concentration in the lungs of smokers versus nonsmokers (Lewis *et al.*, 1972). Alveolar macrophages are scavenger cells in the lungs and are capable of phagocytizing debris which enter the lungs. Therefore, these studies were undertaken to determine whether a soluble cadmium-binding protein is present within the alveolar macrophage. Previous experiments in this laboratory have delineated the toxic effects and interactions of several metals in these cells in culture (Waters *et al.*, 1975a,b,d; Huisingh *et al.*, 1977).

METHODS

Cultures

Alveolar macrophages were obtained by saline endotracheal lavage (Lewis *et al.*, 1972) from New Zealand white rabbits of both sexes and were kept chilled following removal from the animal. One experiment was performed using macrophages obtained from animals treated with *Bacillus Calmette-Guerin* (BCG) to promote an influx of macrophages into the lungs. This technique involved injecting the rabbits in the marginal ear vein with 0.1 mg of heat-killed BCG homogenized in Marcol Oil (Exxon Corp., Charlotte, N.C.) 2-3 weeks prior to sacrifice (Myrvik *et al.*, 1962). There was no apparent difference in cadmium-binding protein isolated from these animals when compared to nonstimulated animals.

The macrophages were centrifuged at 365 g for 12 min and the saline was decanted. Cells were resuspended in an appropriate volume of culture medium to prepare a suspension of 1.0×10^6 cells/ml. The culture medium consisted of Medium 199 (Morgan *et al.*, 1960) with Earle's salts, to which was added 20% fetal bovine serum, 1% sodium monophosphate, 100 units/ml of penicillin-streptomycin, and 100 units/ml of kanamycin. Culture media and supplements were obtained from GIBCO, Grand Island, N.Y. At zero time, $10 \mu\text{M}$ CdCl_2 and $10 \mu\text{Ci}$ of carrier-free ^{109}Cd were added to a 250-ml cell suspension. The cells were then incubated at 37°C in a spinner flask (Wheaton, Millville, N.J.) under an atmosphere of 5% CO_2 . After 24 hr, the cell suspension was placed on ice and cells were collected by centrifugation at 365 g for 15 min at 4°C . The cells were washed three times with ice-cold Ca- and Mg-free phosphate-buffered saline and were frozen as a pellet (-20°C).

Chemicals

Chemicals employed were of analytical grade. Cadmium chloride was obtained from Matheson, Coleman & Bell, Norwood, Ohio. Carrier-free ^{109}Cd was from New England Nuclear Corp., Boston, Mass. Trizma HCl, Trizma Base, and potassium phosphate were from Sigma Chemical Co., St. Louis, Mo. Blue dextran and a column calibration kit containing ribonuclease A, chymotrypsinogen A, ovalbumin, and aldolase were obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N.J.

In order to prevent metal contamination, all solutions were prepared with double-deionized water; sterilization was carried out in a stainless steel autoclave using steam prepared from this water.

Equipment

Teflon-fitted glass columns (2 × 58 cm) for chromatography using Sephadex G-75 (Fine) and Sephadex G-25 were obtained from Pharmacia Fine Chemicals, Inc. Dialysis was performed using Bio-Rad Hollow-Fiber 10 Beaker from Calbiochem, La Jolla, Calif. Electrophoresis was carried out using a Bio-Rad gel electrophoresis cell, Model 150. Flameless atomic absorption spectrophotometry was performed on a Perkin-Elmer Model 306 instrument, with an HGA-2100 accessory. A Packard Auto-Gamma scintillation spectrometer, counting both the X-ray and γ -ray regions, was used to quantify the ^{109}Cd in the fractions.

Preparation of Soluble Cadmium-Binding Protein

Preparation for column chromatography. The basic procedures that were employed in the isolation of this protein were similar to those of Nordberg *et al.* (1974) and Winge *et al.* (1975). All steps in the preparation were performed on ice or at 4°C. Figure 1 illustrates the overall isolation procedure.

The cells were homogenized in a Heidolph homogenizer for 10 min in 1–2 ml of chilled buffer consisting of 0.01 M Tris-HCl and 0.05 M NaCl, pH 7.4. The homogenate was centrifuged for 45 min at 100,000 g at 4°C and the supernatant fraction was removed for determination of total counts of ^{109}Cd . A 0.1-ml aliquot of supernatant was removed for determination of total cadmium by atomic absorption spectrophotometry and total protein was determined using the method of Lowry *et al.* (1951). A blank containing Tris buffer was employed to compensate for colour produced by this compound in the procedure of Lowry *et al.* (1951).

Column Chromatography. The Sephadex column (2 × 58 cm) was packed with Sephadex G-75 (Fine grade) and equilibrated overnight with buffer consisting of 0.01 M Tris-HCl and 0.05 M NaCl, pH 7.4. A flow rate of 20 ml/hr was established. The 100,000 g supernatant fraction was applied to the column and 5-ml fractions were collected. For each fraction eluted from the column, ^{109}Cd radioactivity, absorption at 254 and 280 nm, and total cadmium concentration were determined.

All chromatography was performed in a cold room maintained at 4°C. The column was kept flowing continuously in order to prevent bacterial growth. Each time the column was packed, a void volume determination was performed using blue dextran.

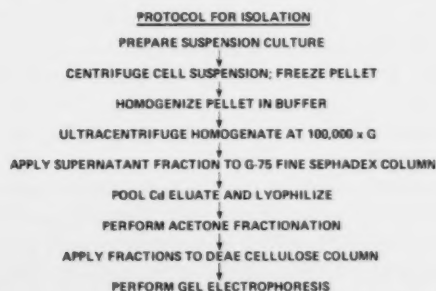


FIG. 1. Flow chart of the protocol employed for the isolation of the cadmium-binding protein from alveolar macrophages.

Lyophilization. Fractions containing cadmium were pooled and lyophilized. The sample was desalted by passing the protein dissolved in 2 ml of deionized water through a Sephadex G-25 column (1.5 × 30 cm) using 0.05 M phosphate buffer, pH 7.8, to elute. The flow rate was 20 ml/hr and 5-ml fractions were collected. The fractions containing cadmium were again pooled and lyophilized.

Acetone fractionation. This lyophilized protein was dissolved in 12 ml of deionized water and the conductivity was checked to insure that it was below 5 mΩ⁻¹/cm. A volume of cold acetone was then added to the protein to make a 40% solution. The mixture was centrifuged at 23,500 g for 2–3 min. The acetone was decanted into another centrifuge tube and the precipitate, if present, was air-dried. The precipitate was then dissolved in 1–2 ml of deionized water. This procedure was repeated to bring the acetone concentration to 60 and 80%. Cadmium, zinc, and total protein were determined for each fraction.

DEAE-Cellulose chromatography. The fraction containing the cadmium-binding protein was dissolved in deionized water and, if necessary, was dialyzed against 0.01 M Tris-HCl buffer, pH 8.6, until the conductivity was 0.1 mΩ⁻¹/cm. The sample was then applied to a 1.9 × 4.0-cm column packed with DEAE-cellulose and equilibrated with 0.02 M Tris buffer, pH 8.6. The sample was eluted with a gradient of 0.01 to 0.4 M Tris-HCl buffer, pH 8.6. Eight-milliliter fractions were collected. Conductivity, ¹⁰⁹Cd, and total cadmium were determined.

Gel electrophoresis. Polyacrylamide gel electrophoresis was performed on the two peaks resolved in the DEAE-cellulose chromatography. Electrophoresis was performed using 7.5% gels by a procedure modified from Jovin *et al.* (1964).

RESULTS

An experiment was performed to determine the kinetics of cadmium uptake during 30 hr of cultivation. Figure 2 depicts the curve obtained. After a lag of 10 hr, cadmium uptake approximately doubled every 4.5 hr over a 20-hr observation period. After 24 hr, the viability of the cells was 92% which was within the control range for macrophages cultured under similar conditions (Waters *et al.*, 1975c). On the basis of this experiment, a 24-hr culture period was selected.

Two additional experiments were designed to insure that the ¹⁰⁹Cd was not simply binding to a component of the fetal calf serum in the media and being concentrated within the macrophages. The cells were cultured in Medium 199 with Earle's salts, without fetal calf serum. The viability was determined to be somewhat lower (83%), however, cadmium-binding material could be isolated on the Sephadex G-75 column. The fetal calf serum was also incubated in the presence of ¹⁰⁹Cd, then passed through the G-75 Sephadex column. In this case, cadmium was detected in the void volume and none was within the expected elution volume.

The 100,000 g supernatant was found routinely to contain approximately 75–85% of the cadmium radioactivity taken up by the cells. The elution profile of the 100,000 g supernatant from Sephadex G-75 column chromatography is seen in Fig. 3. The first protein peak corresponds to the void volume of the column. The second protein peak presumably albumin and other cellular components, has a relative elution volume, V_e/V_o , of 1.22. The third protein peak has a V_e/V_o ratio of 1.87. This latter relative elution volume compares favorably to values obtained by other laboratories for

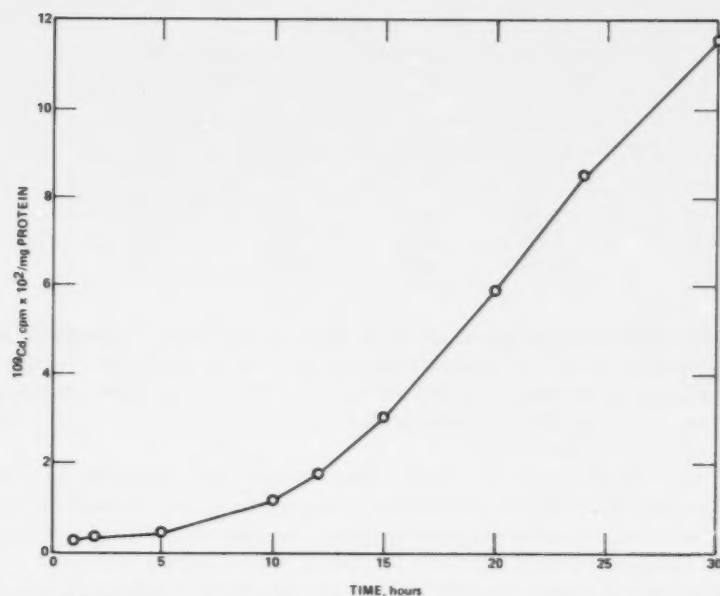


FIG. 2. ^{109}Cd uptake by rabbit alveolar macrophages in suspension culture over a 30-hr period. At each time point, duplicate aliquots, containing approximately 1×10^6 cells, were removed, washed three times with phosphate-buffered saline, lysed with sodium deoxycholate, and counted.

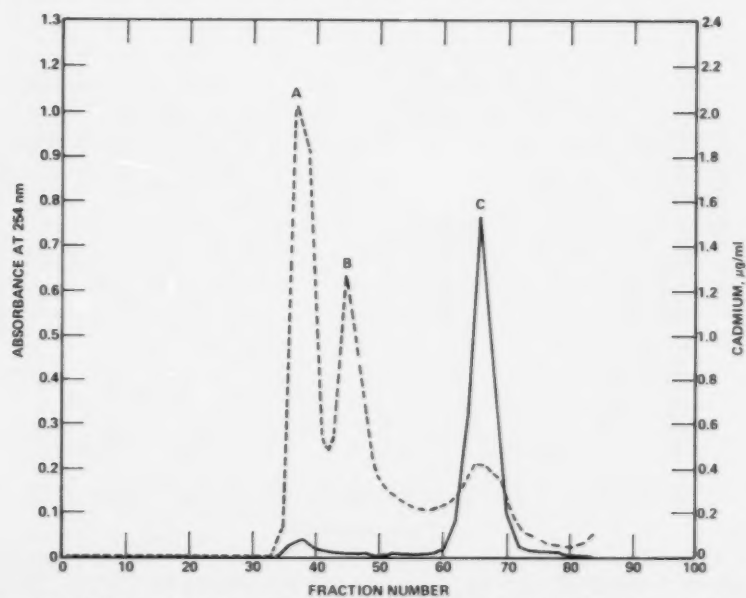


FIG. 3. Cd-binding protein elution profile from a column of Sephadex G-75 Fine. (----) Absorbance at 254 nm; (—) cadmium, micrograms per milliliter, contained in each 5-ml fraction.

TABLE I
ACETONE FRACTIONATION OF Cd-BINDING PROTEIN
SHOWING CONTENT OF TOTAL PROTEIN, CADMIUM,
AND ZINC

Acetone (%)	Total protein (mg/ml)	Cadmium ($\mu\text{g/ml}$)	Zinc ($\mu\text{g/ml}$)
0-40	0.22	0.45	0.11
40-60	5.30	9.29	0.80
60-80	4.00	16.00	0.10

cadmium-thionein (Nordberg *et al.*, 1974; Winge *et al.*, 1975). Furthermore, the third peak contained 86-95% of the total cadmium eluted from the column. The colorimeter indicated that the absorbance at 254 nm was two- to three-fold higher than at 280 nm. Fractions containing cadmium were pooled and lyophilized so that further purification procedures could be performed.

An approximate molecular weight determination was performed by comparing reference proteins of known molecular weights to that of the cadmium binding peak. The relative elution volume was plotted versus the logarithm of the known molecular weight. This plot indicated that the Stokes radius of the impure protein corresponded to that of a globular protein of 11,000 molecular weight. Since metallothionein is known to

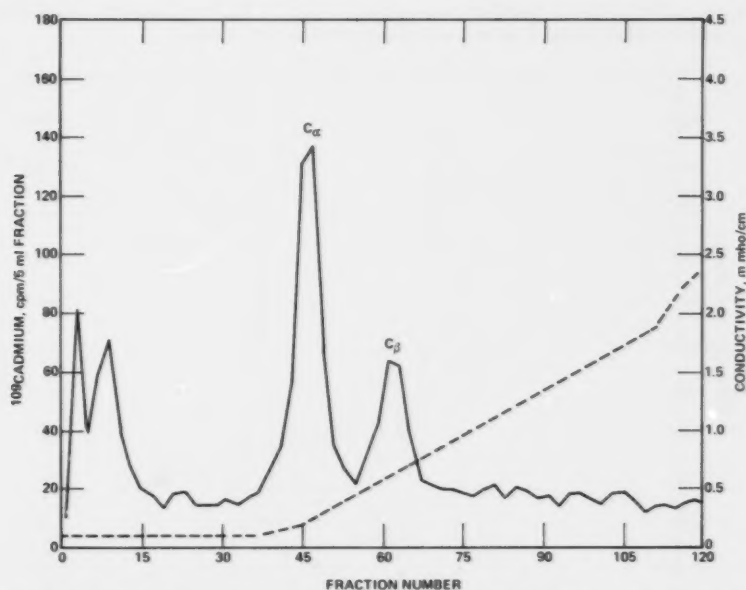


FIG. 4. DEAE-Cellulose chromatography elution profile of the "C" peak isolated by Sephadex G-75 Fine column chromatography. (----) Conductivity, millimohs per centimeter; (—) ^{109}Cd in each fraction.

be asymmetrical, this value undoubtedly overestimates the true molecular weight of the protein.

Acetone fractionation was performed on the pooled fractions derived from Sephadex G-75 chromatography. Total protein, cadmium, and zinc determinations for each of the acetone fractions are shown in Table 1. The major proportion (64–96%) of cadmium-binding protein was found consistently in the 60–80% fraction (Winge *et al.*, 1975). However, in the case illustrated there was some cadmium-binding protein in the 40–

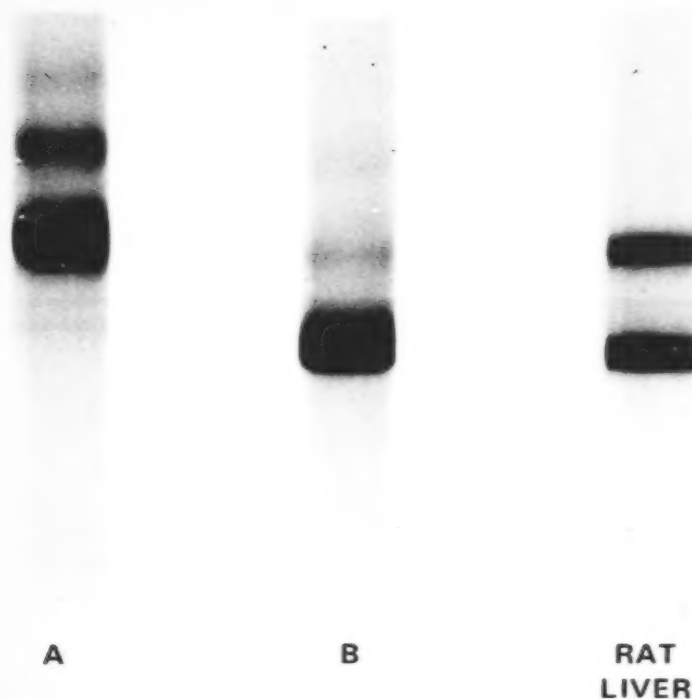


FIG. 5. Disc gel electrophoresis of C_a and C_b peaks obtained from DEAE-cellulose chromatography as compared to a purified sample of rat liver metallothionein.

60% fraction. It is interesting to note that very little zinc was found in any of the acetone fractions.

Both the 40–60 and the 60–80% fractions were combined, applied to a DEAE-cellulose column, and eluted with a gradient of 0.01 to 0.4 M Tris-HCl buffer, pH 8.6. The elution profile is illustrated in Fig. 4. Two major metal-containing peaks were obtained in volumes corresponding to conductivities of 0.25 and 0.63 m Ω^{-1} /cm. The first peak (C_a) and the second peak (C_b) were pooled separately. The samples were then concentrated by lyophilization. Peaks similar to the two peaks which were obtained have been described by other investigators (Winge *et al.*, 1975) with rat liver as a source

of metal-binding protein; however, higher conductivities have generally been observed for peak (A) which corresponds to (C_α) above.

The two forms of metalloprotein cadmium-thionein (C_α and C_β) were subjected to gel electrophoresis using 7.5% polyacrylamide gels. Figure 5 shows the Coomassie blue-stained protein bands. There was poor separation of the two proteins on the DEAE-cellulose column, however, when one compares these gels to a sample of pure

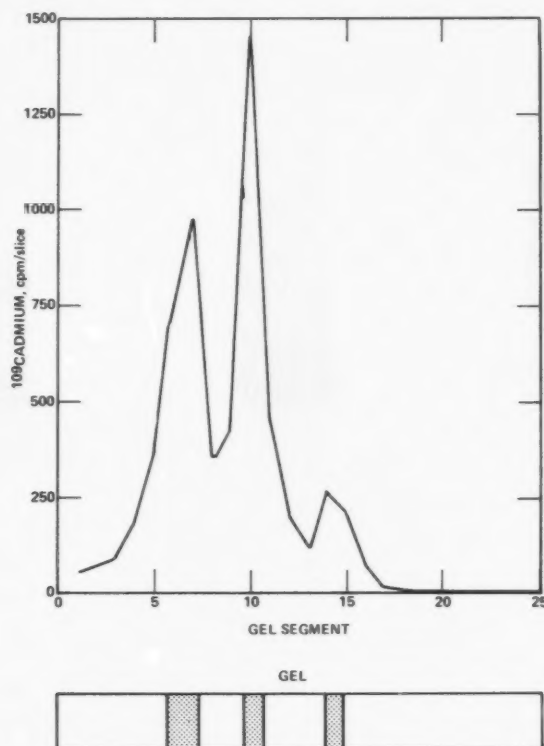


FIG. 6. Distribution of ^{109}Cd on the disc gel electrophoresis. Two gels were prepared. One was sliced into 2.5-mm sections; the other was stained. The graph represents the ^{109}Cd in each section and may be compared to the illustration of the gel segment below.

metallothionein obtained from rat liver (courtesy of Dr. Dennis Winge, Duke University), it may be seen that there were two peaks corresponding to those isolated from rat liver which has been well characterized (Winge *et al.*, 1975). To determine whether the cadmium peaks corresponded to the protein in the gel, an unstained gel was sliced into 2.5-mm segments and each segment was then counted in the gamma counter. The ^{109}Cd profile, as it corresponds with the protein peaks, is illustrated in Fig. 6.

A UV scan was performed on each of the peaks isolated by the DEAE-cellulose chromatography. The A_{230}/A_{280} ratio was 3.0 which is not as high as those values

reported by other investigators (Winge *et al.*, 1975; Nordberg *et al.*, 1974) and is indicative of the presence of contaminating proteins as was seen on gel electrophoresis.

DISCUSSION

This study indicates that a soluble cadmium-binding protein is present in rabbit alveolar macrophages. Under the experimental conditions employed, the amount of this protein is very small when expressed on a per cell basis. This fact prevents further characterization of the metalloprotein isolated from alveolar macrophages *in vitro* until better purification procedures can be developed. Most of the techniques employed represented only slight modifications of the procedures employed by other investigators (Nordberg *et al.*, 1974; Winge *et al.*, 1975) in isolation of metallothionein from liver and kidney where the protein is more abundant. The cadmium-binding protein which has been described has properties similar to those of the well-characterized metallothionein isolated from liver (Winge *et al.*, 1975).

An interesting observation in this study was the apparent lack of zinc in the protein even though the medium contained zinc (0.55 $\mu\text{g/ml}$). A cadmium/zinc ratio of 4.5 is typically reported in metallothionein isolated from rat liver (Piscator, 1964). One could hypothesize that the macrophage has an inherently different metallothionein which selectively binds cadmium. This contrasts with current thought that metallothionein is principally involved in zinc metabolism. One must also consider the possibility that, in an *in vitro* situation, perhaps zinc uptake mechanisms do not function as they do *in vivo*. Further analysis of other Zn-dependent enzymes should indicate whether they too are deficient in zinc in this system. Such investigations may clarify the reason for the apparent lack of Zn in macrophage metallothionein and may indicate a possible function of macrophage metallothionein in zinc metabolism.

Although the role of the macrophage metallothionein with respect to the metabolism of cadmium is unclear, the possibility exists that this system may represent a pulmonary defense mechanism against inhaled cadmium. The results demonstrate that, at extracellular concentrations which do not affect cell viability (Waters *et al.*, 1975c), cadmium enters rabbit alveolar macrophages slowly and binds to the low molecular weight soluble proteins. Additional studies have provided evidence of a threshold concentration for cytolethality and an associated time factor. Hence, the data suggest the presence of limited protective mechanisms which may diminish the cellular toxicity of cadmium on acute exposure. Whether these mechanisms are operative under conditions of long-term low-level exposure *in vivo* is of considerable interest. Furthermore, it is essential to determine whether the biosynthesis of the alveolar macrophage metallothionein is inducible in response to cadmium or zinc exposure in the intact animal. Studies to date in this laboratory have failed to demonstrate induced biosynthesis of the metallothionein by alveolar macrophages *in vitro* or *in vivo*.

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